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Report 2158

ANALYSIS OF CHITIN IN CONTAMINATED FUELS

September 1975

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U. S. ARMY MOBILITY EQUIPMENT RESEARCH AND DEVELOPMENT CENTER
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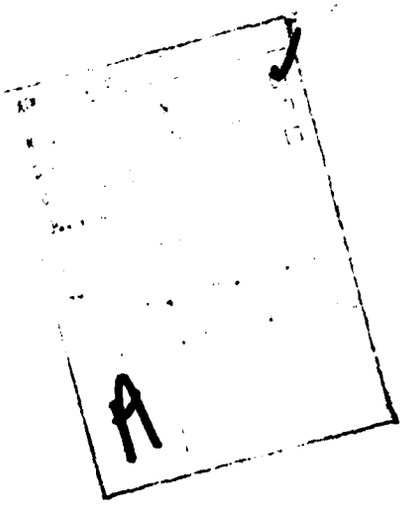

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A test method is described for indicating an early stage of biodegradation (fouling) of hydrocarbon fuels by fungi and for chemically differentiating a massive fungus contamination from a nonbiological one. The test results may be used to differentiate between corrosion caused by fungi and from other origins. The test method is based on the chemical analysis of chitin, a polymer of N-acetylglucosamine, which is a cell wall constituent of most fungi.

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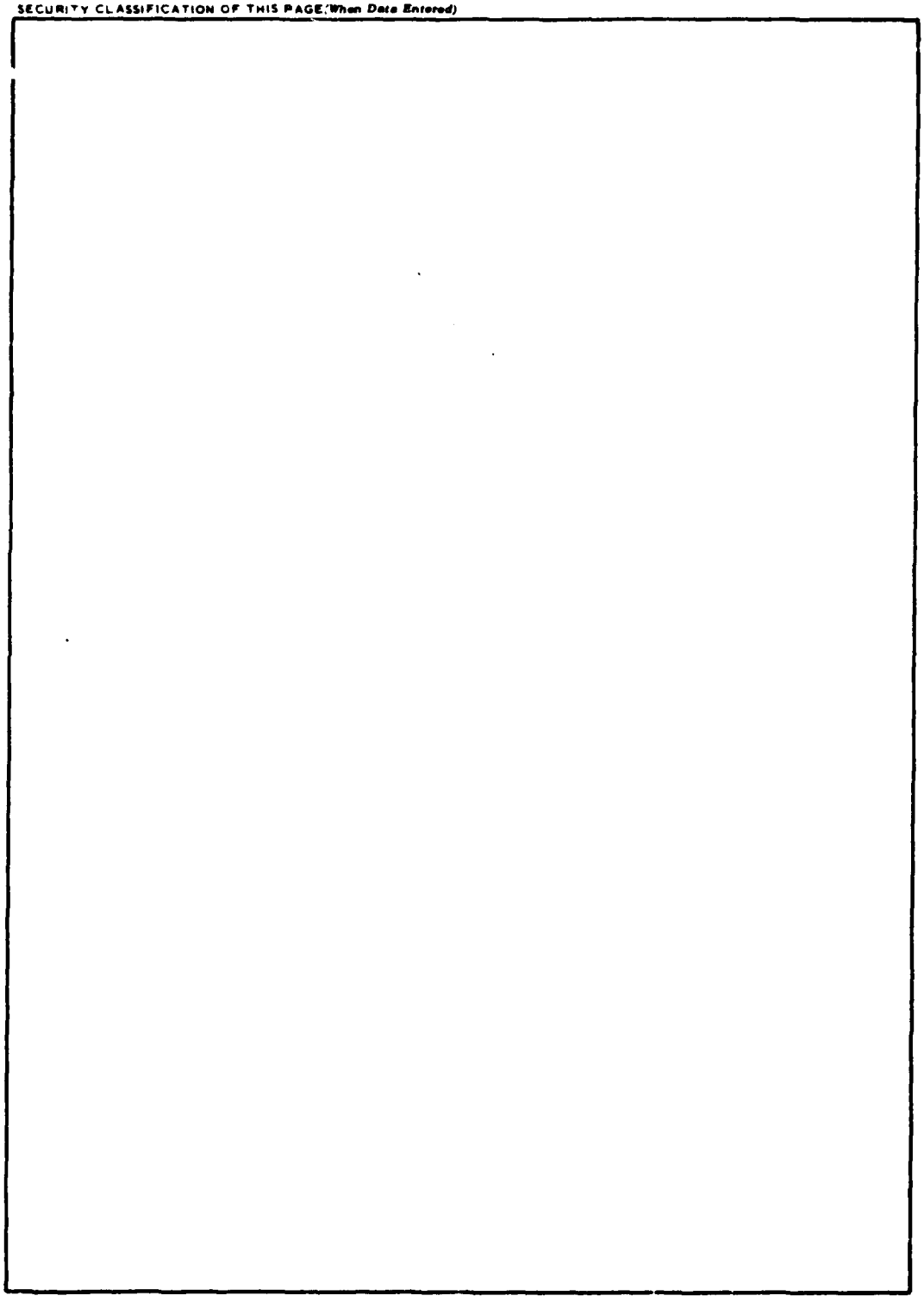

PREFACE

The work was accomplished by G. Ernst, D. A. Emeric, and S. Levine under the direction of Emil J. York, Chief, Materials Engineering Division, Laboratory 4000, USAMERDC. Technical contribution was made by Vincent J. Bagdon, Materials Engineering Division.

CONTENTS

ANALYSIS OF CHITIN IN CONTAMINATED FUELS

I. INTRODUCTION

1. Statement of the Problem. To provide a rapid and convenient chemical analytical technique which may be utilized for early detection of fungus growth in *debris- and non-debris-contaminated* fuel samples.

2. Background. The Armed Forces have utilized fuels in tropical and semi-tropical areas where the optimum conditions for fungus growth during storage and/or field use exist.

An analytical procedure has been developed which could be used as a rapid and convenient control technique for indicating an early stage of biodegradation of hydrocarbon fuels. In addition, this technique may be used to evaluate fuel samples for their service potential during long storage periods and as a diagnostic aid for investigating corrosion problems in the field. This procedure is a modification of a technique by Rondle and Morgan[1] [2] and is based upon the reaction of aminosugars with acetylacetone, in an alkaline medium, to form a chromogen that reacts with Ehrlich's reagent (p-dimethylaminobenzaldehyde-HCl dissolved in ethyl alcohol and concentrated HCl) to produce a pink color. The intensity of the color is proportional to the concentration of the aminosugar.

Chitin, a polymer of glucosamine, is present in the cell wall of most fungi[3] and, thus, if determined quantitatively serves to indicate the degree of fungus infiltration.

II. EXPERIMENTAL PROCEDURE

3. Approach to the Problem. Experimentally contaminate clean JP-5 (to which Bushnell-Haas medium[4] has been added) with *Cladosporium resinae* and incubate until

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[1] L. E. Elson and W. T. J. Morgan, 1933, "A Colorimetric Method for the Determination of Glucosamine and Chondrosamine," Biochem. J. 27: pp. 1824-1828.

[2] C. J. M. Rondle and W. T. J. Morgan, 1955, "The Determination of Glucosamine and Galactosamine," Biochem. J. 61: pp. 586-589.

[3] Jackson W. Foster, 1949, *Chemical Activities of Fungi*, Academic Press, Inc., New York, N.Y., p. 90.

[4] The Bushnell-Haas medium has the following composition:

	g/l distilled water
Magnesium Sulfate	0.2
Calcium Chloride	0.02
Monopotassium Phosphate	1.0
Ammonium Nitrate	1.0
Ferric Chloride	0.05

growth becomes visible. Recover growth from the interface, and determine chitin content. After a procedure has been established, locate and sample a variety of fuel-storage sites. Analyze these samples for chitin, and correlate with standard microbiological techniques.

III. RESULTS

4. Laboratory. Several fuel samples (200 ml of JP-5 enriched with 50 ml of Bushnell-Haas medium) were experimentally contaminated with *C. resinae*. The samples were incubated at 28°C until growth was visible. The samples were filtered through a 1.2-micrometer filter manufactured by the Millipore Corporation, and each residue was analyzed for its chitin content by the Rondle-Morgan method. No glucosamine values (above the sample blank) were obtained. A fuel debris sample obtained from an aircraft fuel (fuel-water interface) was analyzed, and a high glucosamine value was obtained. Microbiological analysis of the debris resulted in the isolation of a fungus belonging to the genus *Paecilomyces*. In spite of the fact that *C. resinae* is frequently the predominant fungus[5] isolated from fuel, we decided, because of the positive (high) glucosamine values obtained from *Paecilomyces*, to investigate the chitin content of other fungi found growing in the fuel-water interface. For this purpose, we selected the following fungus species:[6] *Aspergillus luchuensis, Cladosporium resinae, Paecilomyces varioti, Penicillium janthinellum,* and a mixed culture containing all the fungus species mentioned above. *A. luchuensis* was isolated from a tent in New Guinea. All of the other organisms were originally isolated from fuel.

Fuel-water samples were prepared from JP-5 and a salt-mineral medium. This medium[7] replaced the water and was composed as follows:

	g/l distilled water
KH_2PO_4	2.6
K_2HPO_4	2.2
$NaNO_3$	3.0
$MgSO_4 \cdot 7H_2O$	0.25
Yeast Extract	0.2

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5 R. T. Darby, E. G. Simmons, and B. J. Wiley, 1968, "A Survey of Fungi in a Military Aircraft Fuel Supply System," International Biodeterioration Bulletin 4 (1), pp. 39-41.

6 The fungus cultures were supplied by the Pioneering Research Laboratory, U.S. Army Natick Laboratories, Natick, Massachusetts, 01700. The present address for obtaining cultures is: G. E. Simmons, Natick Laboratories Culture Collection of Fungi (QM), Department of Botany, University of Massachusetts, Amherst, Massachusetts 01002.

7 M. R. Rogers and A. M. Kaplan, 1964, "A Survey of the Microbiological Contamination in a Military Fuel Distribution System," Dev. Indust. Microb. 6, pp. 80-94.

The initial pH was between 6.5 and 7.0.

The samples were incubated until growth could be noticed and then were analyzed for their chitin content. All the fungi with the exception of *C. resinae* gave glucosamine values above the sample blank. It was decided to increase the sample interface volume (by increasing the number of sample bottles which were analyzed) in order to obtain higher values and increased sensitivity. It is interesting to note that *A. luchuensis* and *P. janthinellum* gave the highest glucosamine values, and *C. resinae* gave the lowest glucosamine value (see Table 1).

Table 1. Glucosamine Analysis of Experimentally Contaminated Fuel

Fungi	Glucosamine in Sample (mg)*	
	5 Bottles/Set	1 Bottle/Set
Mixed Culture	6.2	1.3
P. varioti QM 8465	2.4	0.3
P. varioti QM 8009	3.9	0.3
A. luchuensis QM 873	6.2	1.1
P. janthinellum QM 8464	7.3	0.7
C. resinae QM 8013	0.5	negative

* Each set incubated for 7 to 8 days

In order to further increase sensitivity, the number of bottles per set was varied (see Table 2).

Table 2. Glucosamine Analysis of *P. janthinellum* QM 8464

No. of Bottles	Glucosamine in Sample (mg)	
	3 Days' Incubation	6-7 Days' Incubation
10	0.7*	–
5	0.2	7.0
3	0.0	2.4
2	0.0	1.0

*2 days' incubation

Further experiments with a mixed culture consisting of *P. varioti* QM 8465 and QM 8009, *A. luchuensis* QM 873, *P. janthinellum* QM 8464, and *C. resinae* QM 8013 and various time frames indicated that 10 bottles/set was a practical number. The results were as follows:

Time Frame (h)	Glucosamine in Sample (mg)
0	0.0
24	0.0
24*	0.3
27	0.2
45	0.2
48	0.4
72	2.5

* 20 bottles/set; all other samples were 10 bottles/set.

5. **Field.** Samples from the fuel-water interface were collected with a "Golden Thief" sampling device (made by W & W Manufacturing Company, Chicago, Illinois) in presterilized, widemouth glass bottles. Fuel samples collected from different sources were analyzed for their chitin content. It was found that iron interfered with the procedure because of the coloration from iron (III) chloride ($FeCl_3$). Tin (II) chloride ($SnCl_2$) was used to reduce the iron, but this also interfered with normal color development. In order to eliminate the iron interference, the residue was washed with 6N HCl and then rinsed several times with distilled water. Diesel fuel (DF-2) samples with no fungal contamination yielded upon analysis a light-yellow or a light-brown solution, which absorbed strongly at 530 nanometers. These yellow-colored solutions led to high readings and had to be discarded. The presence of even very low fungal levels in DF-2 produced upon analysis the customary pink-colored solution.

Debris-contaminated fuel samples (JP-4 and DF-2) were obtained at different areas at the U.S. Army Mobility Equipment Research and Development Center (USAMERDC) and were analyzed for their chitin content. The results were negative (no chitin). However, microbiological analyses of the fuel samples indicated the presence of fungi. Contaminated fuels (JP-4 and MoGas) from the Crash Rescue Area at Davison Army Airfield in Fort Belvoir, Virginia, were analyzed for their chitin content. The results were also negative. Microbiological analyses of these fuel samples also indicated the presence of fungi.

The negative chitin values obtained by the chemical method as against positive findings (fungi were isolated by microbiological technique) may be explained by the fact that the chemical method is apparently unable to determine chitin in spores. However, chitin can be determined when mycelium is present. In order to hasten the appearance of the mycelium in the fuel if spores are present, sterile Bushnell-Haas solution was added to the field-obtained fuel samples, and the mixture was placed on a shaker for 24 hours prior to filtration and chitin analysis. Diesel fuel samples were analyzed chemically and bacteriologically for the presence of fungi. The results are correlated in Table 3.

Table 3. Correlation of Chemical and Microbiological Methods for Fungal Contamination

Sample No.	Results, Chemical Method	Growth, Microbiological Method	Incubation Period (days)
1	Neg	No	5
2	Neg	No	5
3	Neg	No	5
4	Pos	Yes	3
5	Pos	Yes	3
6*	Neg	No	5

* 20 bottles/set; all other samples were 10 bottles/set.

IV. DISCUSSION

6. **Discussion.** Fuel-water samples that were heavily laden with debris were found to quickly clog the 1.2-micrometer Millipore filter. A Millipore prefilter (AP 20) was helpful but did not solve the "clogging" problems. Filtration of the heavily contaminated fuel-water samples was accomplished by the judicious use of untreated cotton osnaburg cut to fit the Millipore filter holder (see figure on page 6). The residue on the cotton filter was removed for analysis by means of an ultrasonic dismembrator (see analytical procedure in appendix).[8]

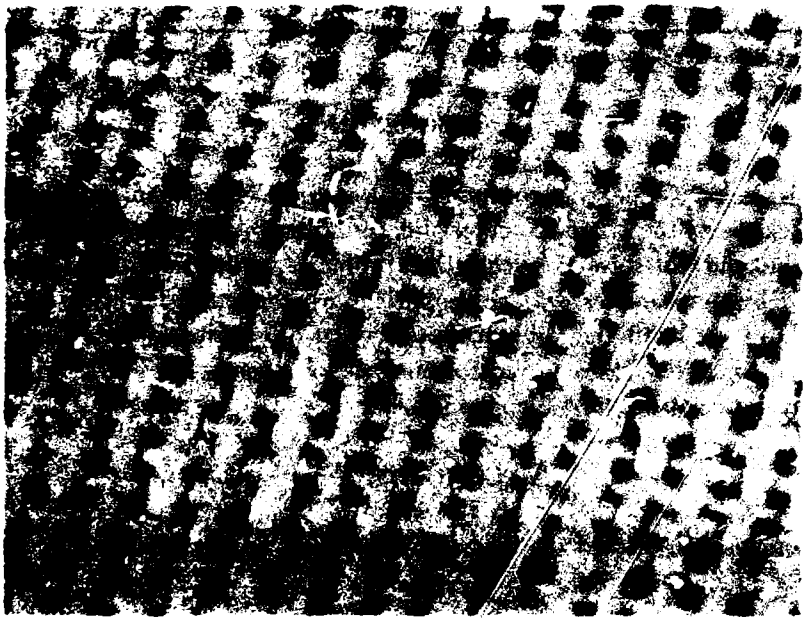
V. CONCLUSIONS

7. **Conclusions.** Based on the work contained in this report, it is concluded that:

a. It is possible to detect low levels of fungus contamination in fuel-water interfaces.

b. A rapid and convenient technique has been established to detect the presence of low levels of fungus growth in the fuel-water interface even when heavily contaminated with inorganic and organic debris.

[8] G. Ernst, D. A. Emeric, and S. Levine, *Quantitative Chemical Essay of Chitin as a Measure of Fungal Infiltration in Cellulosic Materials*, To be Published.



Cotton osnaburg, 40 by 30 yarns/in.^{2}, having a weight of 0.8 g/ft^2.

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APPENDIX

ANALYTICAL PROCEDURE FOR THE DETERMINATION OF CHITIN AS GLUCOSAMINE

1. Equipment and Materials:

a. Apparatus:

(1) Spectrophotometer or colorimeter (wavelength: 530 nanometers)
(2) Heaters
(3) Refluxing apparatus (preferably with ground-glass joints)
(4) 10 ml test tubes graduated in 0.2 ml divisions with ground-glass stoppers
(5) Ultrasonic dismembrator
(6) Shaking apparatus

b. Reagents:

(1) 12N hydrochloric acid
(2) 2N hydrochloric acid
(3) 0.5N hydrochloric acid
(4) 10N sodium hydroxide
(5) 1N sodium hydroxide
(6) Phenolphthalein solution (1%)
(7) 2, 4-pentanedione (acetylacetone). This reagent must be re-distilled every 2 weeks.
(8) 0.5N sodium carbonate
(9) Ethanol (absolute)
(10) Ehrlich's reagent (1.336 grams of p-dimethylaminobenzaldehyde is dissolved in 50 ml of ethanol followed by the addition of 50 ml of 12N hydrochloric acid). Store at -10°C.
(11) Bushnell-Haas medium
(12) Glucosamine hydrochloride (1ml = 8.6 mg glucosamine). Store frozen at -10°C.

2. Procedure.

a. Take at least 10 bottles (bottle dimensions are 5 inches high and 2½ inches wide at the base) of the suspected fuel sample collected from the interface. Add sterile Bushnell-Haas medium to the field-obtained fuel samples. Place the mixture on

a shaker for 24 hours. A matrix control must be analyzed simultaneously with the sample.

b. Filter the fuel sample through a prefilter (Millipore AP2004700) or through cotton osnaburg. If cotton osnaburg is used, dislodge the residue with the ultrasonic dismembrator by passing the probe back and forth across the residue area at least three times.

c. Add an equal volume of 12N hydrochloric acid to the volume of water containing the residue, and transfer the total volume to the refluxing apparatus. Millipore or prefilters containing the filtered residue may be placed directly into 6N hydrochloric acid. In either case, the final volume of the acid refluxing medium should not exceed 50 ml.

d. Reflux for 6 hours.

e. Cool the contents and filter through a Whatman 41-H filter paper or equivalent. Collect the filtrate into a 100-ml volumetric flask.

f. Rinse with distilled water, and bring flask up to volume. (The analysis may be interrupted at this time for a maximum of 24 hours.) A reagent blank and glucosamine must be carried throughout the procedure.

g. A 1.0-ml aliquot is taken from the test solution(s) and pipetted into a 10-ml test tube(s). Analysis must be carried out at least in duplicate.

h. Add a drop of phenolphthalein, and slowly titrate with 10N NaOH until a pink color appears; then, back-titrate with 2N HCl until the pink color is discharged. Back-titrate with 1N NaOH until a pink color appears, and then back-titrate with 0.5N HCl until the pink color is discharged.

i. Pipet 1.0 ml of acetylacetone (1.0 ml of acetylacetone dissolved in 50.0 ml of 0.5N sodium carbonate). Prepare daily. The final volume at this point should be 3.0 ± 0.2 ml; bring to volume with distilled water if necessary.

j. Place a glass tube condenser into a test tube. The condenser must not touch the contents of the test tube. NOTE: An elongated glass tube with one sealed end or a conical test tube filled with water will act as a loose stopper and condenser to prevent the loss of acetylacetone.

k. Place the test tube containing the condenser in a vigorously boiling water bath for 20 minutes.

l. Bring the contents of the test tube to room temperature, and add ethanol (absolute) up to the 9.0-ml mark.

m. Add 1.0 ml of Ehrlich's reagent, and thoroughly mix the contents of the tube.

n. Place the test tube in a 65°C water bath for 10 minutes to accelerate the liberation of carbon dioxide. Bring the test tube to room temperature, and bring up to the 10-ml mark with alcohol if necessary.

o. Spectrophotometer is set to 100% T with a reagent blank at a wavelength of 530 nanometers.

p. Transfer contents to a spectrophotometer cell. Color must be read within ½ hour to avoid error due to fading.

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